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Enhancing the resistance to physical stress in larvae of *Mugil cephalus* by the feeding of enriched *Artemia* nauplii

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Abstract

Striped mullet, *Mugil* cephalus, larvae were first reared exclusively on rotifers until 15 days post-hatching. Larvae were then separated and reared until 35 days post-hatching using four different rearing strategies: Treatment 1=newly hatched Artemia nauplii, Treatment 2=rotifers plus nauplii, Treatment 3=enriched nauplii, and Treatment 4=rotifers plus enriched nauplii. No obvious differences in larval growth and survival were observed between treatments. However, significant differences in the ability to tolerate physical handling were observed. Highest mortalities occurred with larvae reared using Treatment 1. Intermediate percentages of mortality were observed from larvae grown using Treatment 2 and almost no mortalities were observed when employing enriched nauplii (i.e., Treatments 3 and 4).

1. Introduction

A major task in developing a protocol for the artificial propagation of a fish species targeted for culture is the development of a feeding regimen for the larvae. Several impediments are encountered at initial feeding for larval marine fish species, such as the large numbers of food organisms that need to be produced and the size of the food organisms. However, two live foods, the rotifer ***Brachionus plicatilis*** and the nauplii of the brine shrimp ***Artemiu,*** satisfy both the numerical

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and dimensional requirements (Lubzens et al., 1989; Sorgeloos et al., 1991). Recently, the realization that the nutritional quality of both these food organisms can be manipulated to meet the requirements of the larval species being cultured has resulted in major strides in the hatchery production of fish. Enrichment or "boosting" of the fatty acids of both feed organisms have become incorporated into the larval rearing protocols for many fish species (Sorgeloos et al., 1991).

The artificial propagation of striped mullet, *Mugil cephalus*, has been a goal of researchers for over two decades, and yet the seed for stocking into grow-out ponds is obtained exclusively from the wild (Lee and Tamaru, 1988). Similar to other fish species under investigation, significant improvements in growth and survival were not obtained until it was realized that the nutritional requirements (i.e., long-chain *n-3* highly unsaturated fatty acids) of the initial feed (i.e., rotifers) were not being met (Eda et al., 1990; Tamaru et al., 1991, 1993b). The nutritional quality of rotifers is improved by the feeding of the unicellular algal species *Nannochloropsis oculata*. While the cited investigations clearly demonstrate the necessity for these essential fatty acids and that they can be obtained from rotifers that are fed to mullet larvae, the use of enriched *Artemia* nauplii for the rearing of striped mullet has yet to be examined. The use of enriched *Artemia* nauplii for other species has been reviewed by Sorgeloos and Leger (1992).

2. Materials and methods

Source of eggs

Striped mullet (*Mugil cephalus*) broodstock were obtained at The Oceanic Institute maturation facility in Hawaii, USA, between January and March 1992. Females were staged for maturity by ovarian biopsy as validated by Shehadeh et al. (1973) and only females possessing oocytes that averaged $\geq 600\ \mu m$ ($n = 100$) were used for spawning. Induction of spawning was achieved with a two-injection protocol as described by Lee et al. (1987). The first injection involved using 40 mg/kg body weight of carp pituitary homogenate (Stoller Fish Company, Spirit Lake, IA, USA) as the priming injection followed 24 h later with an injection of 100 μg/kg body weight, LHRH-a (des-Gly^{10}-D-Ala^{6} LHRH-ethylamide, Oriental Scientific Instruments Import and Export Corporation, Shanghai, China). All spawnings were conducted with milting males at a sex ratio of 1 female/2 males.

Artemia enrichment

The enrichment medium used in this investigation consisted of menhaden oil (Zapata Haynie Corporation, Reedville, VA, USA) polysorbate emulsifier (Gulf Pacific Industries, Aukland, New Zealand) and tap water. The emulsion was prepared by first heating 200 ml of menhaden oil to a temperature of 50°C on a hot plate with a magnetic stirrer. The heated oil, 200 ml of hot tap water (40–50°C) and 10 ml of emulsifier were then placed into a blender and mixed until the solution took on the consistency of cream. The emulsified oil was then separated

into two 250-ml plastic bottles and stored at 5°C. A new batch of emulsified menhaden oil was made every 3 weeks.

Artemia cysts (San Francisco Bay Brand) were hatched using standard procedures (Sorgeloos et al., 1986). The nauplii were separated, collected into a 66 μm net, and thoroughly rinsed with seawater. The collected nauplii were then placed into 15 liters of seawater and quantified volumetrically. Treatment groups 1 and 2 of mullet larvae were fed newly hatched nauplii directly. The remaining nauplii were stocked at a density of 500 individuals/ml in 10 liters of seawater and vigorously aerated. The emulsified oil was then added to these nauplii to obtain a final concentration of 250 ppm. The nauplii were enriched overnight. After enrichment, the nauplii were collected into a 66 μm nytex collecting net and rinsed with seawater. They were then quantified volumetrically and fed to the larvae in treatment groups 3 and 4.

Fatty acid analysis

Fatty acid profiles of newly hatched and enriched *Artemia* nauplii were determined following the procedures of Folch et al. (1956) and Klopfenstein (1971), with slight modification. The methods are described in detail by Tamaru et al. (1992). Fatty acid methyl esters were quantified by gas chromatography on an HP 5890 gas chromatograph containing a Restek Stabilwax megabore column. In all cases, a known quantity of the C_{17} fatty acid heptadecanoate was added to the original sample and recovery of the fatty acid was measured. Results were corrected for incomplete recovery of heptadecanoate. Analyses with < 80% recovery were repeated. The results were triplicated and are expressed as mg/100 mg dry weight unless otherwise specified. The fatty acids were analyzed statistically with one-way ANOVA (Sokal and Rohlf, 1969).

Larval culture

Culture during the initial 15 days post-hatching was carried out following the protocol described by Eda et al. (1990). Spawned eggs were stocked into a 5000-liter fiberglass rearing tank at a density of 25 eggs/l and at a salinity of 35 ppt. Eggs were allowed to hatch and initial larval densities ranged between 15 and 20 larvae/l. On the second day after hatching, the rotifer *Brachionus plicatilis* (S-type, 110–230 μm lorica length) was stocked at a density of 10–20 rotifers/ml and the unicellular alga, *Nannochloropsis oculata*, was stocked at a density of 300–500 × 10^3 cells/ml. These initial densities were maintained until the start of the experiment. Rotifers were cultured with a batch culture method using N. *oculata* at a density of ≥ 10 × 10^6 cells/ml plus 0.5 g of fresh baker's yeast/ 1 × 10^6 rotifers, respectively as described by Tamaru et al. (1993a). The culture of *N. oculata* has been described by Tamaru et al. (1993a).

The experiment was carried out in 12 × 40-liter fiberglass tanks painted black with epoxy paint (Permalite Plastics Corporation, Newport Beach, CA, USA). Each tank was 36 cm in depth and 40 cm in diameter and equipped with a central drain with a 1-inch PVC standpipe with the sides cut away and replaced with 250 μm nytex screening. The water level of each tank was controlled with a 1-inch

PVC outer standpipe. All tanks received aeration via an air ring around the central standpipe and received continuous water exchange at a rate of 100%/day. No phytoplankton was introduced into the experimental tanks. All tanks were placed in a water bath with flow-through seawater to maintain water temperature ranging from 25.5–28.0°C.

The experiment began by collecting 15-day-old larvae from the 5000-liter larval rearing tank. This was accomplished by first concentrating them With a 17×25 cm aquarium scoop net. The larvae were then scooped out of the rearing tank with plastic pails and placed into the experimental tanks. Each of the experimental tanks was stocked with 200 individuals (i.e., 5 larvae/l).

The experimental design for each trial consisted of four treatment groups and each treatment was triplicated. The treatments consisted of different live foods. These were: Treatment 1 = newly hatched nauplii, Treatment 2 = rotifers plus newly hatched nauplii, Treatment 3 = enriched nauplii, and Treatment 4 = rotifers plus enriched nauplii. In the treatments that included rotifers, the densities were maintained at 10–20 rotifers/ml throughout the course of the experiment. Nauplii (i.e, newly hatched and enriched) were initially stocked into the appropriate tanks at 0.0 1/ml. The densities were subsequently doubled every other day until the end of the experiment. The experiment was carried out for a duration of 20 days after stocking (i.e., 35 days post-hatching). The water quality parameters that were monitored daily during the course of the experiment were temperature (range 25.5–28.0°C), dissolved oxygen (range 5.5–6.2 ppm) and salinity (range 32–35 ppt). No significant differences in these water quality parameters were observed among the treatment tanks [analysis of co-variance (Sokal and Rohlf, 1969), using days as the co-variate].

On the last day of the experiment approximately 30 individuals were scooped from the tank and suspended in the air for a duration of 15 seconds. They were then immediately placed into a 15-liter plastic bucket equipped with aeration and 10 liters of seawater. This was repeated once for each tank. The fry in the buckets were allowed to recuperate for 1 h, at which time the number of dead and live individuals was recorded. Stress resistance is equivalent to the percent survival that occurred after the stress test:

[(number alive in bucket)/total number in bucket] × 100.

The fish remaining in the tanks were then anesthetized with 60 ppm of tricanemethanosulfonate (MS-222, Sigma Chemical Company, St. Louis, MO, USA) and fixed in 2.5% glutaraldehyde (Eastman Kodak Company, Rochester, NY, USA) as described by Oozeki and Hirano (1988).

Survival for each tank was computed by adding the number of the individuals in the stress test and those remaining in the tank and dividing by the total number stocked (i.e., 200) at the beginning of the experiment. Growth was assessed by obtaining the standard lengths from a minimum of 30 individuals from each tank. Individuals were measured with a dissecting microscope equipped with an ocular micrometer. Percent survival and stress resistance were analyzed with one-way ANOVA after arcsine transformation (Sokal and Rohlf, 1969). Standard lengths

were analyzed with one-way ANOVA after log transformation of the data. The entire experiment was repeated with larvae from a separate spawning.

3. Results

The fatty acid profiles of the newly hatched Artemia nauplii and those that were enriched are presented in Table 1. Newly hatched nauplii were found to be lacking in at least two fatty acids (i.e, 22:*n*-11 and 22:6n-3). After enrichment, however, total fatty acids almost doubled. When fatty acids were analyzed individually, it was found that all of them were significantly ($P < 0.05$) elevated after being enriched with the menhaden oil emulsion. Most noticeable was the presence of 22:6n-3 in the enriched nauplii.

The results of the larval-rearing experiments are summarized in Table 2. No significant differences could be detected in percent survival among the treatments. This was consistently observed in both trials. No statistical differences could be detected in standard length among the treatment groups of Trial 1. However, in Trial 2 larvae fed only enriched nauplii were found to be significantly ($P < 0.05$) smaller in comparison to all of the other treatment groups.

The percentage of fry that survived the physical handling of being caught were found to exhibit significant ($P < 0.01$) differences between treatments. Larvae that had been fed only newly hatched nauplii were found to be the most sensitive to the stress test. On the average, approximately 30% of the larvae survived after

Table 1
Fatty acid profiles of newly hatched and enriched *Artemia* nauplii used in the current investigation

Fatty acid	Newly hatched nauplii	Enriched nauplii
14	0.06±0.00	0.36±0.01
16	0.82±0.06	1.77±0.07
16:1n-7	0.32±0.02	0.75±0.04
18	0.53±0.03	0.77k0.03
18:1n-9	1.52f0.07	2.38±0.09
18:2n-6	0.49±0.09	0.64±0.02
18:3n-3	1.94±0.08	2.57f0.09
18:4n-3	0.28±0.02	0.47±0.01
20:1n-9	0.05±0.00	0.12±0.00
20:4n-6	0.17f0.01	0.20±0.00
20:5n-3	0.36±0.02	1.02±0.05
22:1*n*-11	n.d.	0.02±0.00
22:6n-3	n.d.	0.50±0.01
Total	6.55±0.23	11.6±0.40

Values are expressed in terms of mg/100 mg dry weight and are the means ± standard deviations from three replicates.
n.d. = not detected.

Table 2
Survival, growth and stress resistance of 35-day post-hatched larval striped mullet in response to various feeding regimens

Treatment	n	Survival (%)	SL (mm)	Stress resistance (%)
Trial 1				
Hatched nauplii	3	66.6 ± 7.6	17.9 ± 1.5	27.0 ± 3.6[a]
Rotifers + hatched nauplii	3	70.0 ± 4.4	16.95 1.0	55.0 ± 8.5[b]
Enriched nauplii	3	65.3 ± 1.2	16.0 ± 0.6	98.7 ± 1.9[c]
Rotifers + enriched nauplii	3	68.3 ± 3.2	16.5 k0.6	95.3 ± 2.5[c]
Trial 2				
Hatched nauplii	3	65.0 ± 12.6	16.2 ± 2.4[b]	21.8 54.5[a]
Rotifers + hatched nauplii	3	71.8 ± 8.3	16.0 ± 1.0[b]	55.0 ± 8.5[b]
Enriched nauplii	3	52.0 ± 12.0	14.9 ± 0.6[a]	99.2 ± 1.2[c]
Rotifers + enriched nauplii	3	62.9 ± 9.0	15.8 ± 1.4[b]	99.6 ± 0.6[c]

The values are means ± standard deviations from three replicates. Values within each column of each trial that do not share a superscript are significantly ($P < 0.05$) different.

being physically stressed. Larvae that had been fed a combination of rotifers plus newly hatched nauplii were significantly ($P < 0.01$) more resilient than the first treatment group. In this case more than 50% of the larvae could recover from being physically handled. However, almost all of the larvae recovered after being caught when they were fed enriched nauplii regardless of whether they were provided rotifers. The identical trend in stress resistance was also found with larvae from the second trial.

4. Discussion

One of the main objectives of developing larval rearing strategies is the establishment of a feeding regimen that will result in optimal growth, survival and health of the fish larvae. For rearing mullet larvae, rotifers have always been used as the initial food (Nash et al., 1974; Kraul, 1983; Eda et al., 1990). With the exception of using enriched *Artemia* nauplii, the present treatments simulated the major live food types and feeding strategies that have been employed for rearing of striped mullet larvae beyond 15 days post-hatching. Although artificial diets have reportedly been used in the rearing protocol for striped mullet (Eda et al., 1990), the current investigation focused only on the live food components.

The enrichment process for *Artemia* nauplii used in this investigation was found to significantly elevate all of the fatty acids found in the nauplii. The values are similar to those reported in other reports using other lipid sources (i.e. Super-Selco at 300 ppm) (Kraul et al., 1993). Most notable is the tripling of 20:5n-3 as well as infusion of 22:6n-3. Both of these have been implicated to be essential for larval growth and development in a number of fish species (Watanabe et al.,

1983; Dhert et al., 1990; Sorgeloos et al., 1991; Sorgeloos and Leger, 1992; Watanabe, 1993; Kraul et al., 1993).

Resulting growth and survival of the striped mullet larvae in the treatments reported here were similar and it appears that all treatments provided sufficient amounts of n-3 highly unsaturated fatty acids (HUFAs). In contrast, stress resistance was significantly improved when larvae were provided a source of enriched feed (i.e., rotifers and/or *Artemia* nauplii). The correlation of acquisition of increased stress resistance with increased n-3 HUFAs in the diet with little or no effects on growth and survival has also been reported with other fish and crustacean species (Tackaert et al., 1989; Dhert et al., 1990, 1992; Kraul et al., 1993). This correlation has led to the use of resistance of various stressors (i.e., physical, environmental, chemical) as another criterion to evaluate hatchery products, in addition to the traditional measures of survival and growth (Tackaert et al., 1989; Dhert et al., 1992). Stress resistance provides the hatchery operator/investigator with another avenue of evaluating hatchery operating procedures. For example, the physical sorting of highly cannibalistic species (e.g., mahimahi, *Coryphaena hippurus*) (Ako et al., 1993) is the major means of reducing mortalities due to cannibalism. Acquisition of resistance to physical handling can be used to determine the earliest time when sorting by size can commence. Furthermore, acquired resistance to physical stress in mahimahi reportedly prevents catastrophic losses of larvae in response to other stressors (i.e., temperature) (Kraul et al., 1993). For striped mullet larvae, resistance to handling stress rather than survival and growth has been used to determine the end-point of a larval rearing trial when other treatments (e.g., elevated water temperature) are utilized to shorten the duration of its larval rearing phase (Tamaru et al., 1993a). Based on the acquisition of stress resistance, the rearing time was shortened by 7 days which ultimately resulted in a significant reduction in production costs.

The feeding of only newly hatched nauplii from 15–55 days post-hatching (i.e., Treatment 1) was practiced during earlier investigations (Nash et al., 1974). The convention of using rotifers for the duration of the rearing trial in addition to *Artemia* nauplii (i.e., Treatment 2) was established by Eda et al. (1990). It was felt that having two feed types (i.e., small and large) during this transitional period resulted in the reported improvements in overall larval survival. However, it has recently been demonstrated that larval mullet growth and survival during the initial 15 days post-hatching is significantly affected by the nutritional quality of the rotifers cultured on N. *oculata*, because of its high content of n-3 HUFAs (Tamaru et al., 1991, 1993b). With the use of enriched *Artemia* the reason for using rotifers for the duration of the rearing practice is now uncertain.

The continued use of rotifers in the rearing process is a major issue with regard to the costs of producing mullet larvae because of the large amount for labor involved to produce phytoplankton and rotifers. If only survival and growth were used to evaluate the treatments, the results from the current investigation would indicate that there is no advantage in using rotifers for the duration of its rearing. However, if stress resistance is included in evaluating the various treatments, it is clear that there are some benefits in providing rotifers enriched with N. *oculata*

for the duration of the rearing trial if *Artemia* nauplii are not enriched. It would appear that the improved stress resistance is the result of the larvae obtaining appropriate nutritional requirements from these rotifers. This is consistent with the demonstration that larval growth and survival during the 15 days post-hatching are dependent on the nutritional quality of rotifers they were fed (Tamaru et al., 1991, 1993b).

The implications of the present results are that the general health and well-being of the mullet larvae had been significantly improved by the feeding of enriched *Artemia* nauplii. Including the enrichment of nauplii in the other established hatchery practices should insure that a higher quality seed will be produced from the hatchery. This improvement in the quality of the larvae occurs whether or not rotifers are provided throughout the course of the experiment. The results indicate that the improvement in stress resistance is directly the result of an improvement in the fatty acid complement of the enriched nauplii, which is consistent with a number of observations with other cold- and warmwater marine fish species (Lavens et al., 1991). The present work does not determine which of the fatty acids is contributing to stress resistance because all of them were significantly elevated during the enrichment process. However, results from other investigations implicate docosahexaenoic acid (22:6*n*-3) as the fatty acid that confers stress resistance in fish (Kraul et al., 1993; Watanabe et al., 1993).

The issue of whether rotifers should be used throughout the rearing of striped mullet larvae appears to require further investigation. Although there were no statistical differences in survival observed among all treatments and for both trials, the average survival was always a few percentage points higher than when larvae were provided rotifers throughout the experiment. In practical terms, the cost of producing rotifers for the duration of the rearing trial (i.e., including phytoplankton culture) must be weighed against the production of a few more fry. Since it is estimated that labor costs account for 51.1% of the total production costs of mullet fry (Leung et al., 1991), meaningful results can only be obtained if this question is evaluated on a large scale and it must also address the use of artificial diets which 20-day post-hatched larvae will begin to accept (Eda et al., 1990).

The results from the current investigation, however, clearly demonstrate that mullet larvae acquire the ability to tolerate physical stress associated with being handled when provided with enriched *Artemia* nauplii during the course of their rearing and that enriched nauplii should be included in the rearing practice of mullet larvae.

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